Sarcolipin uncouples hydrolysis of ATP from accumulation of Ca^{2+} by the Ca^{2+}-ATPase of skeletal-muscle sarcoplasmic reticulum

Wendy S. SMITH, Robert BROADBRIDGE, J. Malcolm EAST and Anthony G. LEE[1]

Division of Biochemistry and Molecular Biology, School of Biological Sciences, University of Southampton, Southampton SO16 7PX, U.K.

Sarcolipin (SLN) is a small peptide found in the sarcoplasmic reticulum of skeletal muscle. It is predicted to contain a single hydrophobic transmembrane α-helix. Fluorescence emission spectra for the single Trp residue of SLN suggest that SLN incorporates fully into bilayers of dioleoylphosphatidylcholine, but only partially into bilayers of phosphatidylcholines with long (C_{22} or C_{24}) fatty acyl chains. The fluorescence of SLN is quenched in bilayers of dibromostearoylphosphatidylcholine, also consistent with incorporation into the lipid bilayer. SLN was reconstituted with the Ca^{2+}-ATPase of skeletal-muscle sarcoplasmic reticulum. Even at a 50:1 molar ratio of SLN/ATPase, SLN had no significant effect on the rate of ATP hydrolysis by the ATPase or on the Ca^{2+}-dependence of ATP hydrolysis. However, at a molar ratio of SLN/ATPase of 2:1 or higher the presence of SLN resulted in a marked decrease in the level of accumulation of Ca^{2+} by reconstituted vesicles. The effect of SLN was structurally specific and did not result from a breakdown in the vesicular structure or from the formation of non-specific ion channels. Vesicles were impermeable to Ca^{2+} in the absence of ATP in the external medium. The effects of SLN on accumulation of Ca^{2+} can be simulated assuming that SLN increases the rate of slippage on the ATPase and the rate of passive leak of Ca^{2+} mediated by the ATPase. It is suggested that the presence of SLN could be important in non-shivering thermogenesis, a process in which heat is generated by hydrolysis of ATP by skeletal-muscle sarcoplasmic reticulum.

Key words: calcium pump, coupling, reconstitution, thermogenesis.

INTRODUCTION

P-type ATPases couple the hydrolysis of ATP to the movement of ions across a biological membrane. The Na^+/K^+-ATPase and H^+/K^+-ATPase are isolated from membranes as $\alpha\beta$ dimers, in which the large α subunit is the catalytic subunit and the small β subunit is of unknown function. In contrast, the Ca^{2+}-ATPase (sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase 1, or SERCA1 isoform) is isolated from skeletal-muscle sarcoplasmic reticulum (SR) as a single polypeptide of molecular mass 110 kDa, corresponding to the α subunit of the Na^+/K^+- or H^+/K^+-ATPases [1]. As an ATP-driven pump, the Ca^{2+}-ATPase must, with high efficiency, couple the hydrolysis of ATP to the vectorial transport of Ca^{2+} across the membrane; the mechanism of the coupling process is such that two Ca^{2+} ions are transported for each ATP molecule hydrolysed [1]. The single polypeptide chain of the Ca^{2+}-ATPase is certainly able to transport Ca^{2+} across a membrane since microsomal preparations of COS cells expressing the Ca^{2+}-ATPase are able to accumulate Ca^{2+} [2]. Similarly, the purified Ca^{2+}-ATPase, reconstituted into sealed lipid vesicles, is able to accumulate Ca^{2+} driven by the hydrolysis of ATP [3–7]. Nevertheless, the early literature on Ca^{2+}-ATPase contains many references to a small 'proteolipid', defined as a protein extractable into chloroform/methanol [8–10] which, under some conditions, co-purifies with the Ca^{2+}-ATPase [11]. In 1992 Wawrzynow et al. [12] purified sufficient quantities of the proteolipid for sequencing and showed that the proteolipid, which they named sarcolipin (SLN), contained just 31 residues, with a 7-residue hydrophilic N-terminal domain, a 19-residue hydrophobic transmembrane α-helical domain and a 5-residue hydrophilic C-terminal domain. The protein has now been cloned from human, rabbit and mouse [13]. These studies show an important relationship between SLN and phospholamban (PLN), a small hydrophobic peptide found in cardiac muscle which interacts with the SERCA2a isoform of the ATPase, controlling its activity [13,14]. PLN contains a hydrophilic N-terminal domain of about 30 residues linked to a transmembrane domain containing a single transmembrane α-helix [15]. PLN, when unphosphorylated, binds to the Ca^{2+}-ATPase and inhibits it; phosphorylation of PLN by Ca^{2+}/calmodulin-dependent or cAMP-dependent protein kinases leads to dissociation of PLN from the ATPase and expression of the full activity of the ATPase [15]. Odermatt et al. [13] have compared the sequences of the hydrophobic domains of SLN and PLN and identified a common motif, LXXNFXXXLIXXXL, which suggests that SLN and PLN are homologous proteins. SLN is expressed in high amounts in some fast-twitch skeletal muscles [13], but not in others [16], and is expressed at only low levels in cardiac muscle, where high levels of PLN expression are found [13].

Odermatt et al. [14] have reported that co-expression of SLN and SERCA1 Ca^{2+}-ATPase in COS cells leads to a slightly reduced affinity of the Ca^{2+}-ATPase for Ca^{2+} and a slightly

Abbreviations used: di(C14:1)PC, dimyristoleoylphosphatidylcholine; di(C16:1)PC, dipalmitoleoylphosphatidylcholine; di(C18:1)PC, dioleoylphosphatidylcholine; di(C22:1)PC, dierucoylphosphatidylcholine; di(C24:1)PC, dinervonylphosphatidylcholine; di(Br_2C18:0)PC, dibromostearoylphosphatidylcholine; di(C18:1)PA, dioleoylphosphatidic acid; FCCP, carbonyl cyanide *p*-trifluormethoxyphenylhydrazone; PLN, phospholamban; OG, β-D-octyl glucoside; $C_{12}E_8$, octa(ethylene glycol)-n-dodecyl ether; SLN, sarcolipin; C9A-SLN, SLN with Cys-9 replaced by Ala; 3TR-SLN, SLN with the three Thr residues replaced by Ala; SR, sarcoplasmic reticulum; SERCA1, sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase 1; E2PCa_2, Ca^{2+}-bound, phosphorylated intermediate of the ATPase.

[1] To whom correspondence should be addressed (e-mail agl@soton.ac.uk).

increased value for the maximum activity. In these assays Ca^{2+} accumulation by microsomal preparations was measured in the presence of oxalate to precipitate Ca^{2+} within the lumen of the microsomes. Here we have studied the effects of synthetic SLN when reconstituted with the Ca^{2+}-ATPase of rabbit skeletal-muscle SR. We detected no significant effect of SLN on the rate of ATP hydrolysis by the ATPase or on the Ca^{2+}-dependence of ATPase activity, but we did find that the presence of SLN leads to reduced levels of accumulation of Ca^{2+} by the Ca^{2+}-ATPase, through effects on leak and slippage pathways. We suggest that increased rates of leak and slippage of Ca^{2+} could be important for heat production by the SR in non-shivering thermogenesis.

MATERIALS AND METHODS

Dimyristoleoylphosphatidylcholine [di(C14:1)PC], dipalmitoleoylphosphatidylcholine [di(C16:1)PC], dioleoylphosphatidylcholine [di(C18:1)PC], dieicosenoylphosphatidylcholine [di(C20:1)PC], dierucoylphosphatidylcholine [di(C22:1)PC], dinervonylphosphatidylcholine [di(C24:1)PC] and dioleoylphosphatidic acid [di(C18:1)PA] were from Avanti Polar Lipids, and [^{3}H]dipalmitoylphosphatidylcholine was from New England Nuclear. β-D-Octyl glucoside (OG) and octa(ethylene glycol)-n-dodecyl ether ($C_{12}E_8$) were from Sigma and Calbiochem, respectively. Dibromostearoylphosphatidylcholine [di(Br_2C18:0)PC] was prepared by bromination of di(C18:1)PC, as described in East and Lee [17]. SR was prepared from rabbit skeletal muscle as described in Dalton et al. [18]. Purified ATPase was obtained from SR as described in East and Lee [17]. Concentrations of ATPase were estimated using a specific absorption coefficient of 1.2 $l \cdot g^{-1} \cdot cm^{-1}$ for a solution in 1% (v/v) SDS [19].

Rabbit muscle SLN (MERSTRELCLNFTVVLITVILIWLLVRSYQY) was synthesized using t-butoxycarbonyl chemistry with Merrifield or 4-(oxymethyl)phenylacetic acid aminomethylpolystyrene (PAM) resin [20]. The peptide was cleaved from the resin with HF and purified on an LH20 column in order to remove salts, using methanol as a solvent. We also synthesized mutant SLNs, with Cys-9 replaced by Ala (C9A-SLN) and with the three Thr residues replaced by Ala (3TR-SLN). Electrospray-ionization MS was used to characterize the peptides.

Reconstitution into lipid bilayers

SLN (20 nmol of peptide) was reconstituted into lipid vesicles by mixing SLN and lipid at the desired molar ratio (usually 1:100) in chloroform/methanol (2:1, v/v), followed by drying under vacuum to a thin film. Samples were then suspended in 400 μl of buffer (20 mM Hepes/1 mM EGTA, pH 7.2) by sonication in a bath sonicator for 5–10 min, followed by dilution into buffer (2.5 ml) for fluorescence measurements. Fluorescence spectra were recorded using an SLM-Aminco 8000C fluorimeter, with excitation at 280 nm.

Reconstitution with the Ca^{2+}-ATPase

SLN (143 nmol) and di(C18:1)PC, usually at a SLN/lipid molar ratio of 1:40, were mixed in the required molar ratio in chloroform/methanol (2:1, v/v) and dried under vacuum to a thin film. Samples were then resuspended in 400 μl of buffer [10 mM Hepes, pH 8.0, containing 15% (w/v) sucrose, 5 mM $MgSO_4$ and 12 mg/ml potassium cholate] by sonicating for 5–10 min in a bath sonicator. ATPase (0.29 mg) in a volume of 10–20 μl was added to the sonicated lipid/peptide samples and the mixtures were left to equilibrate at room temperature for 10 min and kept on ice until use. Samples were then diluted 170-fold into the ATPase assay media. ATPase activities were determined at 25 °C using a coupled enzyme assay in a medium containing 40 mM Hepes, pH 7.2, 100 mM KCl, 5 mM $MgSO_4$, 2.1 mM ATP, 1.1 mM EGTA, 0.41 mM phosphoenolpyruvate, 0.15 mM NADH, 7.5 units of pyruvate kinase and 18 units of lactate dehydrogenase. The reaction was initiated by the addition of an aliquot of a 25 mM $CaCl_2$ solution to a cuvette containing the ATPase and the other reagents to give the required concentration of free Ca^{2+}. Free concentrations of Ca^{2+} were calculated by using the binding constants of Ca^{2+}, Mg^{2+} and H^+ for EGTA given by Godt [21].

For measurements of Ca^{2+} accumulation a reconstitution method based on that of Levy et al. [6] was used, as described by Dalton et al. [22]. SLN and lipids were mixed in chloroform/methanol, dried and resuspended in buffer A (10 mM Pipes/100 mM K_2SO_4, pH 7.1) by sonication in a bath sonicator. OG was added to give a final concentration of 40 mM. SR was solubilized in buffer A containing $C_{12}E_8$ (6 mg/ml) and 0.1 mM $CaCl_2$. The solubilized SR was mixed with the lipid sample, usually to give a 5000:1 molar ratio of lipid/ATPase. Detergent was removed by addition of four aliquots of washed SM2 Bio-Beads (mesh size 20–50), as described by Levy et al. [6], to give a preparation of sealed vesicles. Accumulation of Ca^{2+} by the reconstituted vesicles was measured at 25 °C using Antipyrylazo III to monitor the external Ca^{2+} concentration. The absorption difference between 720 nm and 790 nm was recorded using an SLM-Aminco dual-wavelength spectrophotometer. The assay buffer used was 10 mM Pipes/100 mM K_2SO_4/5 mM $MgSO_4$, pH 7.1, containing 10 μM Antipyrylazo III and a protein concentration of 0.02 mg/ml. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added to a concentration of 0.25 μM to make the vesicles permeable to H^+. The system was calibrated by the incremental addition of Ca^{2+} to a final concentration of 120 μM prior to the addition of ATP to initiate uptake.

For experiments trapping fluorescein in the lumens of reconstituted vesicles, fluorescein (1 mM) was included in the original buffer. Untrapped fluorescein was removed by passing the reconstituted vesicles through two 5 ml columns of coarse Sephadex G-50. Samples were diluted 25-fold into buffer (10 mM Pipes, pH 4.0/100 mM K_2SO_4) and the fluorescence was recorded, exciting fluorescence at 481 nm and recording fluorescence intensity at 507 nm. When required, vesicles were made leaky by addition of 1.2 mM $C_{12}E_8$.

Vesicles were also reconstituted using the procedure of Racker and Eytan [10]. A 9:1 molar ratio of di(C18:1)PC/di(C18:1)PA (30 μmol) was mixed in chloroform/methanol with the required concentration of SLN, dried down and resuspended in 1 ml of 0.4 M potassium phosphate buffer, pH 7.5, containing 16 mg of potassium cholate. The sample was sonicated to clarity in a bath sonicator, and SR was added to give a final concentration of 1 mg of protein/ml. The sample was then dialysed overnight at 4 °C against 0.4 M potassium phosphate buffer.

Labelling of SLN with pyrene maleimide

The single Cys residue in SLN was labelled by incubation of SLN with a 1:2 molar ratio of *N*-(1-pyrene)maleimide (Molecular Probes) in dimethylformamide for 24 h at room temperature in the dark. Labelled SLN was precipitated by addition of ether and freeze-dried. Using an absorption coefficient of 36000 $M^{-1} \cdot cm^{-1}$ for pyrene, the labelling ratio was determined to be 1:1. The structure of the labelled peptide was confirmed using MS.

Labelling of SLN with FITC

SLN was synthesized with an additional N^{α}-fluoren-9-ylmethoxycarbonyl-Lys-N^{ϵ}-t-butoxycarbonyl at the N-terminus. While it was still resin-bound, the peptide was treated with trifluoroacetic acid [50% (v/v) in dichloromethane] to remove the butoxycarbonyl protecting group from the ϵ amino group of the Lys. The resin-bound peptide was then reacted with 5 molar equivalents of FITC in dimethylformamide containing 3% (v/v) di-isopropylethylamine. After 24 h, the fluoren-9-ylmethoxycarbonyl protecting group was removed using 20% (v/v) piperidine in dimethylformamide. The peptide was cleaved from the resin using HF and purified on an LH20 column. The stoichiometry of peptide labelling was determined by measuring the absorption at 490 nm of the peptide in methanol containing KOH. Using an extinction coefficient of 80000 $M^{-1} \cdot cm^{-1}$ for FITC, the labelling ratio was determined to be 1:1.

Sucrose-density-gradient centrifugation

Lipid samples were prepared as described above for measurements of Ca^{2+} uptake, with an SLN/ATPase molar ratio of 5:1, but containing [^{3}H]dipalmitoylphosphatidylcholine at a molar ratio with di(C18:1)PC of $1:1 \times 10^5$. Samples were loaded on to sucrose gradients containing the following solutions of sucrose (w/w) in buffer A: 2.5, 5, 10, 15, 20 and 30%. The 30% sucrose solution also contained 0.05% Triton X-100. Samples were centrifuged at 80000 *g* for 18 h at 4 °C. Fractions (1 ml) were taken and assayed for lipid and protein by, respectively, liquid-scintillation counting and protein assay (modified Lowry assay from Sigma). For determination of SLN content, FITC-labelled SLN was used instead of SLN and the FITC-SLN content of the fractions was determined by measuring fluorescein fluorescence with excitation and emission wavelengths of 490 and 520 nm, respectively.

Simulations

Simulations of Ca^{2+} accumulation by reconstituted vesicles were performed using the program FACSIMILE (UKEA), as described in [22].

RESULTS

Reconstitution of SLN into lipid bilayers

Residues 8–26 of SLN are uncharged and hydrophobic and thus probably constitute a transmembrane α-helix. The fluorescence properties of the Trp residue at position 23 of SLN can be used to study the interaction of SLN with lipid bilayers. SLN was incorporated into lipid bilayers by mixing SLN with lipid in organic solvent, removing the solvent and hydrating the mixture. The fluorescence emission spectrum of tryptophan is environmentally sensitive, the emission maximum moving to a shorter wavelength with decreasing solvent polarity [23]. Further, fluorescence intensities of hydrophobic Trp-containing peptides in water are very low, probably as a result of aggregation of the peptide [24]. The fluorescence intensity for SLN incorporated into di(C14:1)PC was comparable with that in methanol, whereas that of SLN in water was very low (Figure 1), consistent with incorporation of SLN into the lipid bilayer. The fluorescence emission spectrum of SLN incorporated into di(C14:1)PC was centred at 326 nm (Figure 1), indicating a very hydrophobic environment for the tryptophan residue, consistent with a transmembrane arrangement for SLN in the bilayer. The fluorescence intensities for SLN in di(C14:1)PC and di(C18:1)PC were very similar, but those in di(C22:1)PC and di(C24:1)PC were significantly lower, suggesting only partial incorporation of SLN into these thicker bilayers.

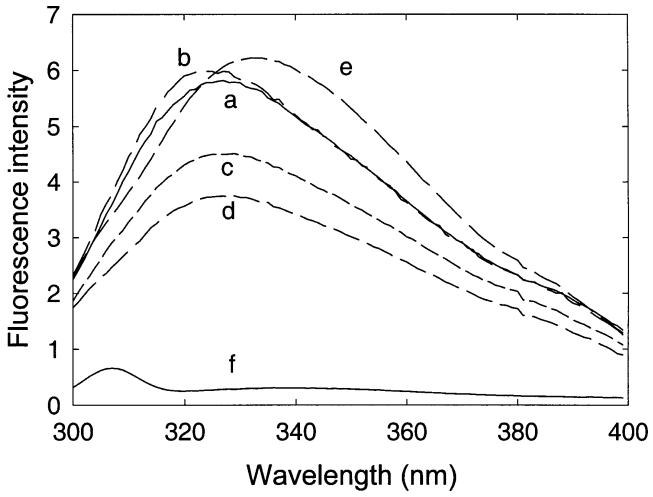


Figure 1 Fluorescence emission spectra for SLN

Shown are the fluorescence emission spectra for SLN in (a) di(C18:1)PC, (b) di(C14:1)PC, (c) di(C22:1)PC and (d) di(C24:1)PC. The SLN/lipid ratio was 1:100. Also shown are the emission spectra for SLN in (e) methanol and (f) water. For all spectra the concentration of SLN was 1.7 μM.

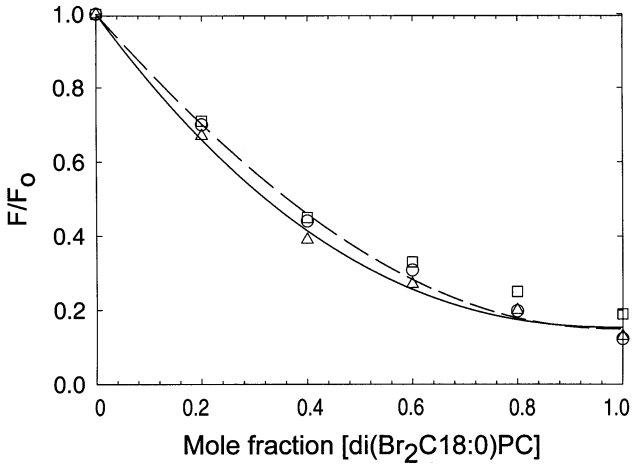


Figure 2 Fluorescence intensity for SLN in mixtures containing di(Br_2C18:0)PC

The experimental points show fluorescence intensities for SLN in mixtures of di(Br_2C18:0)PC with (○) di(C14:1)PC, (□) di(C16:1)PC or (△) di(C18:1)PC. The solid and broken lines show fits to the data for mixtures with di(C18:1)PC and di(C14:1)PC, respectively, with the parameters given in the text. The line of best fit to the data for mixtures with di(C16:1)PC is very similar to that shown for di(C18:1)PC.

Incorporation of SLN into lipid bilayers was confirmed by observation of quenching of the SLN Trp fluorescence by di(Br_2C18:0)PC (Figure 2). The fluorescence intensity for SLN incorporated into bilayers of di(Br_2C18:0)PC at a lipid/SLN molar ratio of 100:1 was 13% of that in di(C18:1)PC, demonstrating highly efficient quenching of the Trp by the bromine-containing fatty acyl chains. The fluorescence intensity of SLN in mixtures of di(C18:1)PC and di(Br_2C18:0)PC decreases with

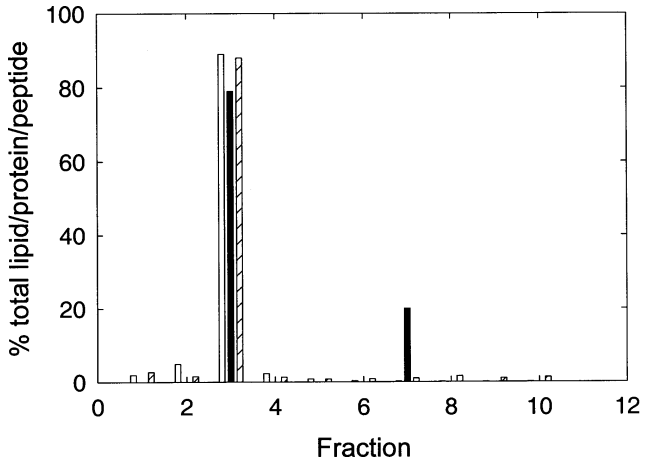


Figure 3 Sucrose-gradient analysis of Ca^{2+}-ATPase reconstituted with SLN

A sample of Ca^{2+}-ATPase reconstituted with SLN in di(C18:1)PC at a molar ratio of Ca^{2+}-ATPase/SLN/di(C18:1)PC of 5000:5:1 was separated on a discontinuous sucrose gradient from 30% to 2.5% sucrose. Fractions (1 ml) were taken and analysed for lipid (open bars) and for total protein using a modified Lowry assay (black bars). The experiment was repeated under identical conditions but with FITC-labelled SLN rather than with SLN, and samples were analysed for FITC-labelled SLN by fluorescence analysis (hatched bars).

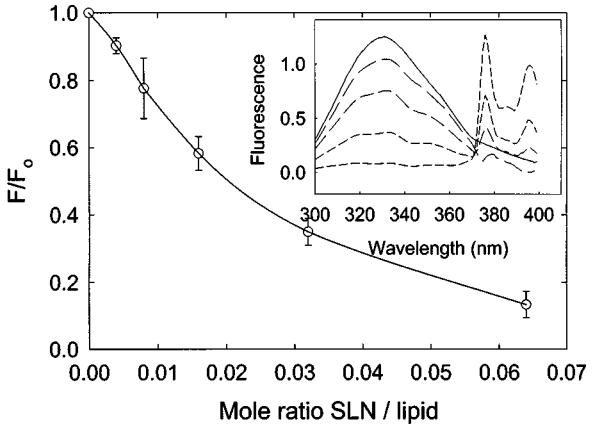


Figure 4 Effect of pyrene-labelled SLN on Trp fluorescence of the Ca^{2+}-ATPase

The Ca^{2+}-ATPase was reconstituted with pyrene-labelled SLN in di(C18:1)PC at a molar ratio of Ca^{2+}-ATPase/di(C18:1)PC of 1:500 and the given molar ratios of SLN/di(C18:1)PC, corresponding to molar ratios of Ca^{2+}-ATPase/SLN of 1:0, 1:2, 1:4, 1:8, 1:16 and 1:32. Trp fluorescence intensity is plotted relative to that in the absence of SLN, using fluorescence excitation and emission wavelengths of 280 and 330 nm, respectively. The insert shows difference fluorescence emission spectra of Ca^{2+}-ATPase plus SLN in di(C18:1)PC minus SLN alone in di(C18:1)PC. Spectra (from top to bottom) correspond to molar ratios of Ca^{2+}-ATPase/SLN of 1:0, 1:4, 1:8 and 1:32. For all experiments the buffer was 20 mM Hepes/1 mM EGTA, pH 8.0.

increasing content of di(Br_2C18:0)PC (Figure 2). Fluorescence quenching in mixtures of di(Br_2C18:0)PC and non-brominated phospholipids was fitted to the equations given in [24], giving values for n, the number of sites around the protein from which a Trp residue can be quenched, and K, the relative binding constant of di(Br_2C18:0)PC with respect to the non-brominated phospholipid. The data fitted to an n value of 2.3 ± 0.16 and relative binding constants of 1.1 ± 0.1 and 1.2 ± 0.1 in di(C16:1)PC and di(C14:1)PC, respectively (Figure 2). The value of n compared favourably with a value of 2.7 estimated for simple peptides of the type $KKGL_nWL_mKKA$ in mixtures with di(Br_2C18:0)PC [24]. The relative lipid-binding constants changed little with changing bilayer thickness, as found for other transmembrane peptides [24,25].

Table 1 Effect of SLN and mutant SLNs on Ca^{2+}-ATPase activity

The ATPase was reconstituted at di(C18:1)PC/peptide/ATPase molar ratios of 2000:50:1. Values are means ± S.D. from between 4 and 7 experiments.

System	pCa value for 50% maximal activity	Maximal activity (i.u./mg of protein)
di(C18:1)PC	6.71 ± 0.01	4.2 ± 0.2
SLN	6.67 ± 0.04	3.9 ± 0.1
3TR-SLN	6.63 ± 0.04	3.9 ± 0.1
C9A-SLN	6.58 ± 0.11	4.4 ± 0.5

Reconstitution of SLN with Ca^{2+}-ATPase

The Ca^{2+}-ATPase was reconstituted into sealed vesicles suitable for measurements of Ca^{2+} accumulation by mixing lipid and SLN in OG with SR dissolved in $C_{12}E_8$, followed by removal of detergent with Bio-Beads. Samples of SLN reconstituted with Ca^{2+}-ATPase in this way were subject to discontinuous sucrose-gradient centrifugation (Figure 3). As shown, $\approx 80\%$ of the lipid and protein were found together at the 2.5–5% sucrose interface, showing that the protein and lipid had reconstituted. The experiment was repeated with FITC-labelled SLN, allowing a determination of the SLN content of the fractions. The FITC-labelled SLN was also found to be located at the 2.5–5% sucrose interface (Figure 3), confirming that the Ca^{2+}-ATPase and SLN had reconstituted into the lipid bilayer.

To confirm that the Ca^{2+}-ATPase and SLN had co-reconstituted into the lipid bilayers, we studied the effect of pyrene-labelled SLN on the tryptophan fluorescence of the Ca^{2+}-ATPase. As shown in Figure 4, incorporation of increasing amounts of pyrene-labelled SLN caused a decrease in tryptophan fluorescence as a result of energy transfer from tryptophan to pyrene. These experiments show that, as expected, the Ca^{2+}-ATPase and SLN were contained in the same membranes.

Effect of SLN on ATPase activity

The Ca^{2+}-ATPase can be reconstituted with peptides by simply mixing the Ca^{2+}-ATPase with a solution of di(C18:1)PC and peptide in cholate, followed by dilution in buffer to re-form the membranes [26]. This procedure is advantageous for measurements of ATPase activity in that it produces unsealed membrane fragments in which all ATPase molecules have access to added ATP. We have found that the minimum molar ratio of phospholipid/peptide for reconstituting hydrophobic peptides into a bilayer is $\approx 15:1$ [24,26]. In these experiments we used a molar ratio of di(C18:1)PC/SLN of 40:1. At an SLN/ATPase molar ratio of 50:1, SLN resulted in only a 10% decrease in ATPase activity, measured at a maximally stimulating concentration of Ca^{2+} (10 μM), and had no significant effect on the affinity of the Ca^{2+}-ATPase for Ca^{2+}, as given by the concentration of Ca^{2+} showing 50% maximal activity (Table 1). Similarly, 3TR-SLN and C9A-SLN had no significant effect on maximal activities or on apparent affinities for Ca^{2+} (Table 1). As described below, SLN in these molar ratios to Ca^{2+}-ATPase had

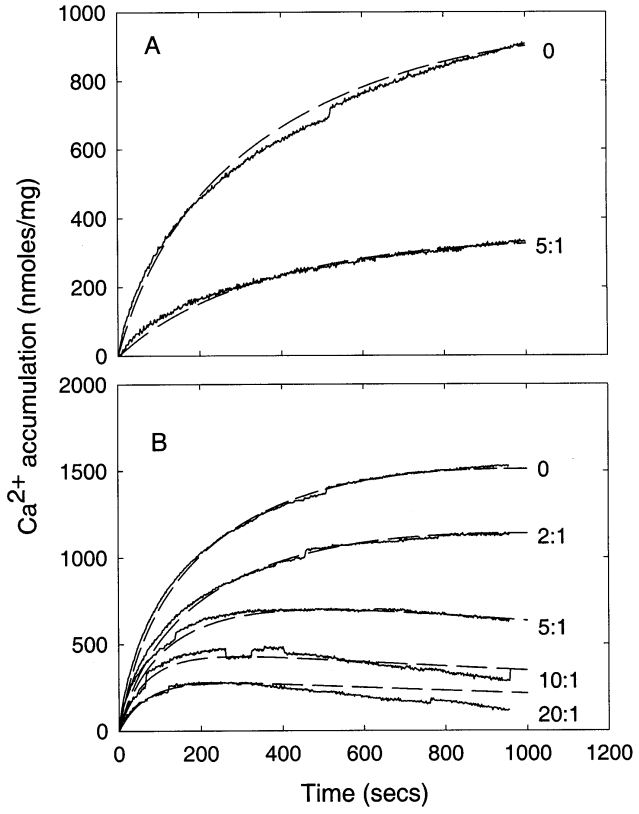


Figure 5 Effect of SLN on ATP-dependent accumulation of Ca^{2+} by reconstituted vesicles

Shown is ATP-dependent accumulation of Ca^{2+} by reconstituted vesicles containing (**A**) di(C18:1)PC or (**B**) a 9:1 molar ratio of di(C18:1)PC/di(C18:1)PA. The vesicles contained SLN at the given molar ratios of SLN/ATPase. In each case, accumulation of Ca^{2+} was initiated by addition of 0.8 mM ATP. Samples contained 0.02 mg of ATPase/ml at a lipid/ATPase molar ratio of 5000:1, in 10 mM Pipes, pH 7.1/100 mM K_2SO_4/5 mM $MgSO_4$/0.25 μM FCCP. The initial concentration of Ca^{2+} was 120 μM. The broken lines show the results of simulations of the accumulation of Ca^{2+}, as described in the text, with concentrations of active, outwardly oriented ATPase of (**A**) 0.03 μM and (**B**) 0.04 μM and the rate constants for slippage and leak given in Table 3.

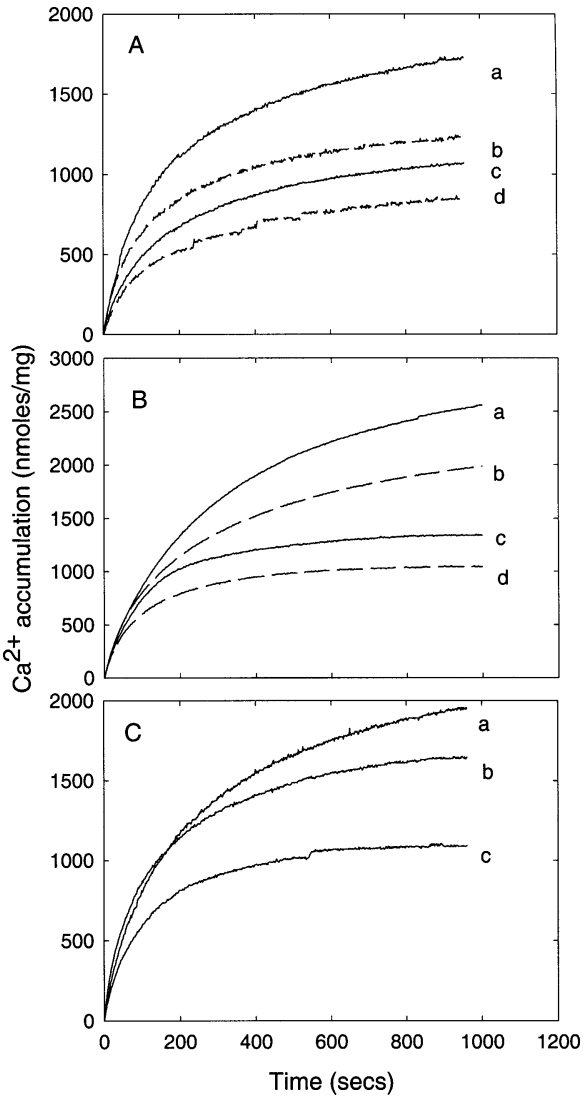


Figure 6 ATP-dependent accumulation of Ca^{2+} under a variety of conditions

ATP-dependent accumulation of Ca^{2+} is shown for vesicles containing a 9:1 molar ratio of di(C18:1)PC/di(C18:1)PA. (**A**) The effect of FCCP. Uptake is shown for reconstituted vesicles in the absence of SLN with (a) or without (b) 0.25 μM FCCP, and in the presence of SLN at an SLN/ATPase molar ratio of 5:1, with (a) or without (c) 0.25 μM FCCP. (**B**) The effect of luminal phosphate. Uptake is shown for reconstituted vesicles in the absence of SLN with (d) or without (b) 50 mM luminal phosphate, and in the presence of SLN at an SLN/ATPase molar ratio of 5:1, with (c) or without (d) 50 mM luminal phosphate. (**C**) Uptake is shown for reconstituted vesicles in the absence of SLN (a) or the presence of 3TR-SLN (b) or C9A-SLN (c), at a molar ratio of peptide/ATPase of 5.1. All other conditions are as described in the legend to Figure 5.

very large effects on accumulation of Ca^{2+} driven by the Ca^{2+}-ATPase. The Ca^{2+}-ATPase was also reconstituted with di(C18:1)PC and SLN at molar ratios of di(C18:1)PC/SLN/ATPase of 2000:100:1 and 500:50:1. Even at these higher molar fractions of SLN, the maximum observed decrease in the pCa value for 50% activity was only 0.1, with no more than a 15% inhibition of maximal ATPase activity (results not shown).

Effects of SLN on accumulation of Ca^{2+}

As reported previously, uptake of Ca^{2+} by sealed vesicles containing the Ca^{2+}-ATPase produced by the Bio-Beads method was relatively low if the vesicles contain only di(C18:1)PC, but was increased considerably if the vesicles contain 10 mol% di(C18:1)PA (Figure 5). The presence of SLN led to a decrease in the level of accumulation of Ca^{2+} in both the absence and presence of di(C18:1)PA, with the effect increasing with the molar ratio of SLN to ATPase, such that uptake was very low at a molar ratio of 20:1 (Figure 5). The effect of SLN was structurally specific. Reconstitution with the simple transmembrane α-helix $KKGL_7WL_9KKA$ had no effect on accumulation of Ca^{2+} (results not shown). Reconstitution with C9A-SLN had the same effect as SLN itself (Figure 6C), as did FITC-labelled SLN (results not shown), but the effects of 3TR-SLN were very small (Figure 6C), suggesting an important role for the Thr residues.

The Ca^{2+}-ATPase was also reconstituted with PLN; PLN had no significant effect on accumulation of Ca^{2+} by vesicles containing di(C18:1)PC and di(C18:1)PA at a molar ratio of PLN/ATPase of 5:1. However, accumulation of Ca^{2+} was slow at a molar ratio of 50:1 (Figure 7A), consistent with the reported inhibition of ATPase activity at high molar ratios of PLN/ATPase [26]. PLN at a PLN/ATPase molar ratio of 10:1 also

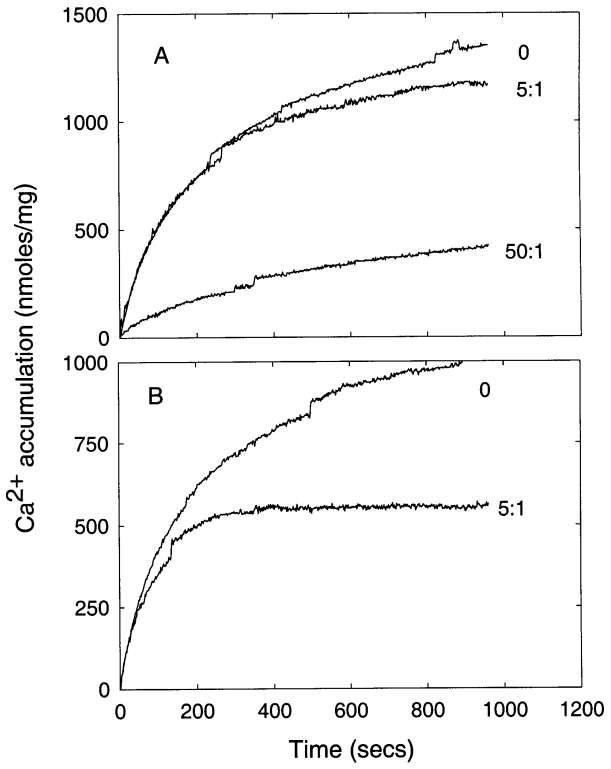


Figure 7 Effect of PLN and method of reconstitution on ATP-dependent accumulation of Ca^{2+} by reconstituted vesicles

(**A**) Shown is the accumulation of Ca^{2+} by reconstituted vesicles containing a 9:1 molar ratio of di(C18:1)PC/di(C18:1)PA and PLN at the given molar ratios of PLN/ATPase. In each case accumulation of Ca^{2+} was initiated by addition of 0.8 mM ATP. Samples contained 0.02 mg of ATPase/ml at a lipid/ATPase molar ratio of 5000:1, in 10 mM Pipes, pH 7.1/100 mM K_2SO_4/5 mM $MgSO_4$/0.25 μM FCCP. The initial concentration of Ca^{2+} was 120 μM. (**B**) Shown is the accumulation of Ca^{2+} by vesicles of di(C18:1)PC/di(C18:1)PA reconstituted as in [10] at the given molar ratio of SLN/ATPase, at a molar ratio of lipid/ATPase of 3000:1. The luminal phosphate concentration of the vesicles was 0.4 M.

had no effect on accumulation of Ca^{2+} by vesicles of di(C18:1)PC (results not shown).

The above experiments were performed in the presence of FCCP. The Ca^{2+}-ATPase acts as a Ca^{2+}/H^+-ATPase, and since the membrane is impermeable to H^+, addition of FCCP results in an increase in the level of accumulated Ca^{2+} (Figure 6A), as reported by Levy et al. [6]. This same increase in the level of accumulation of Ca^{2+} on addition of FCCP is seen in the presence of SLN (Figure 6A), implying that the vesicle membrane must also be impermeable to H^+ in the presence of SLN. The addition of valinomycin to collapse any membrane potential had very little effect in the presence of FCCP for reconstituted vesicles either with or without SLN (results not shown), showing that no large membrane potentials affecting accumulation of Ca^{2+} are generated under these conditions.

To test for the possible importance of leak pathways for Ca^{2+} out of these vesicles, accumulation of Ca^{2+} was studied for vesicles reconstituted in buffer containing 50 mM phosphate. Phosphate in the lumen of the reconstituted vesicles would be expected to complex with Ca^{2+} ions pumped into the lumen, with $Ca_3(PO_4)_2$ precipitating when the solubility limit for $Ca_3(PO_4)_2$ is exceeded, leading to increased levels of accumulation of Ca^{2+} [22]. As shown in Figure 6(B), the presence of luminal phosphate lead to increased levels of Ca^{2+} accumulation, the effect of

Table 2 ATPase activities for reconstituted vesicles

ATPase activities were measured using the experimental conditions described in the Materials and methods section for the ATPase reconstituted with a 9:1 molar ratio of di(C18:1)PC/di(C18:1)PA. The molar ratio of lipid/peptide/ATPase was 5000:5:1. When added, the $C_{12}E_8$ concentration was 0.8 mg/ml. Means ± S.D. are shown.

	ATPase activity (i.u./mg of protein)	
Peptide	$-C_{12}E_8$	$+C_{12}E_8$
–	1.2 ± 0.1	3.1 ± 0.3
SLN	1.4 ± 0.3	3.1 ± 0.3
C9A-SLN	1.4 ± 0.1	3.0 ± 0.2
3TR-SLN	1.2 ± 0.1	3.0 ± 0.4

phosphate being smaller in the presence of SLN than in its absence. The smaller effects of phosphate in the presence of SLN suggest that luminal concentrations of Ca^{2+} are lower in the presence of SLN than in its absence, consistent with an increased leak rate in the presence of SLN.

We also reconstituted the ATPase using the procedure of Racker and Eytan [10]. Uptake levels in the presence of 0.4 M phosphate were less than for vesicles reconstituted by the method of Levy et al. [6] in the absence of phosphate (Figure 7B). Nevertheless, the presence of SLN again results in a decrease in the level of Ca^{2+} accumulation (Figure 7B).

SLN does not form non-specific channels in the membrane

One possible explanation for the reduced level of Ca^{2+} accumulation observed in the presence of SLN is that SLN either breaks down the vesicle membrane structure or forms non-specific channels in the membrane. However, measurements of ATPase activity for the reconstituted vesicles containing SLN show that the vesicle structure must still be intact. As shown in Table 2, ATPase activities for reconstituted vesicles measured with ATP in the external medium approximately doubled on addition of sufficient $C_{12}E_8$ to make the vesicles leaky to ATP. Thus the reconstitution procedure results in incorporation of ATPase molecules into the membrane with a close to random orientation, so that only about half of the ATPase molecules have their ATP-binding sites facing the external medium, and the vesicles remain intact and impermeable to ATP in the presence of SLN.

To establish whether SLN formed non-specific ion channels in the membrane, we studied the permeability of the vesicles to H^+. Fluorescein was trapped within reconstituted vesicles and the change in fluorescence intensity with changing external pH was measured. The presence of -OH and $-CO_2H$ groups on fluorescein make its fluorescence emission pH-sensitive; addition of KOH to a solution of fluorescein at acid pH results in a large increase in fluorescence intensity [27]. Addition of KOH to reconstituted vesicles resulted in an immediate increase in fluorescence intensity due to the small amount of contaminating fluorescein in the external medium; the fluorescence intensity then remained constant, showing that the vesicles were impermeable to H^+ (Figure 8). Addition of $C_{12}E_8$ to make the vesicles leaky resulted in a further increase in intensity due to ionization of the fluorescein previously trapped in the vesicle lumens (Figure 8). The fluorescence responses in the presence of SLN at a 5:1 molar ratio of SLN/ATPase were the same as in the absence of SLN, showing that SLN did not make the membrane permeable to H^+ (Figure 8).

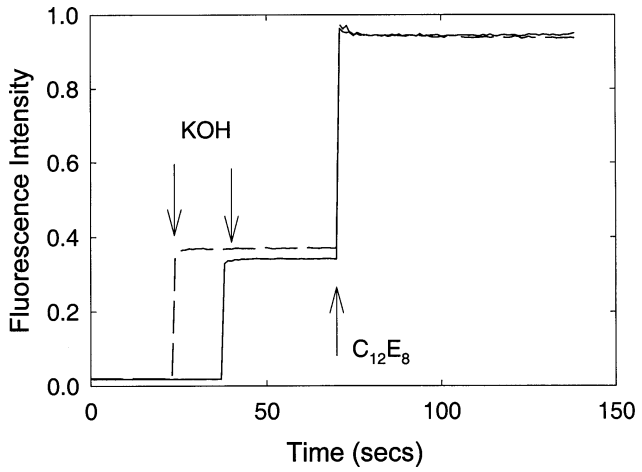


Figure 8 Reconstituted vesicles are impermeable to H^+

Shown are the fluorescence responses of vesicles reconstituted in the presence of fluorescein (0.1 mM) at pH 4.0 to the addition of KOH, to raise the external pH to 10, and $C_{12}E_8$ (1.8 mM), to make the vesicles permeable. The vesicles contained a 9:1 molar ratio of di(C18:1)PC/di(C18:1)PA with a molar ratio of phospholipid/ATPase of 5000:1. The solid line shows responses of vesicles in the absence of SLN and the broken line shows the responses for vesicles in the presence of SLN at an SLN/ATPase molar ratio of 5:1. Following addition of KOH at the times marked by the arrows, the immediate increase in fluorescence intensity corresponded to ionization of contaminating fluorescein in the external medium. Subsequent addition of $C_{12}E_8$ allowed ionization of fluorescein trapped in the lumens of the vesicles.

Reconstituted vesicles show only a very slow leak of Ca^{2+} in the absence of ATP

The rate of simple passive leak of Ca^{2+} from reconstituted vesicles was determined by allowing vesicles to accumulate Ca^{2+} in the presence of ATP and glucose and then adding hexokinase to remove unreacted ATP (Figure 9). Addition of hexokinase to buffer containing ATP and glucose resulted in a small increase in the concentration of free Ca^{2+} in the medium (Figure 9), probably attributable to release of Ca^{2+} from the small amount of CaATP formed under these conditions, the Ca^{2+} being released as the ATP is converted into ADP. Addition of hexokinase to reconstituted vesicles resulted in only a very slow additional release of Ca^{2+}, in either the presence or absence of SLN, which could be estimated to be $\approx 5 \times 10^{-3}$ $\mu M \cdot s^{-1}$ and $\approx 14 \times 10^{-3}$ $\mu M \cdot s^{-1}$ in the absence and presence of SLN, respectively (Figure 9). The accumulated Ca^{2+} could, however, be released on addition of the ionophore A23187 (Figure 9). We conclude that the rate of passive leak of Ca^{2+} is slow in the absence of ATP, in both the absence and presence of SLN.

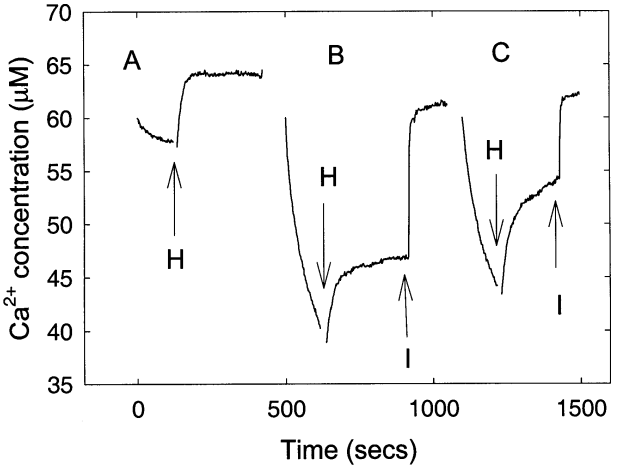


Figure 9 Passive release of Ca^{2+} in the absence of ATP

Vesicles were reconstituted with a 9:1 molar ratio of di(C18:1)PC/di(C18:1)PA, in the absence (**B**) or presence (**C**) of SLN at a lipid/SLN/ATPase molar ratio of 5000:5:1. The buffer was 10 mM Pipes, pH 7.1/100 mM K_2SO_4/5 mM Mg^{2+}, containing 12.5 mM glucose. In (**A**) the buffer also contained 0.6 mM ATP, and hexokinase (H; 56 units) was added at the time marked by the arrow. The increase in cytoplasmic Ca^{2+} was probably due to the release of Ca^{2+} from CaATP as the ATP was converted to ADP; addition of hexokinase to buffer in the absence of ATP resulted in no change in the cytoplasmic concentration of Ca^{2+}. In (**B**) and (**C**), Ca^{2+} accumulation by the reconstituted vesicles (0.021 mg of protein/ml) was initiated by addition of ATP (0.6 mM), followed by addition of hexokinase (H) and then A23187 (I; 15 μg).

DISCUSSION

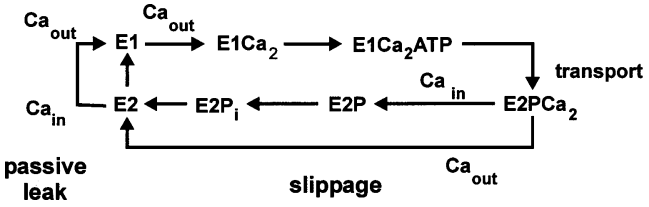
SLN is expressed at high levels in human fast-twitch skeletal muscle, but only at low levels in cardiac muscle, where levels of PLN are high [13]. This, combined with sequence similarities between the hydrophobic domains of SLN and PLN, suggests that SLN could play a role in regulating SERCA1 Ca^{2+}-ATPase in skeletal muscle in some way analogous to the role of PLN in regulating SERCA2 Ca^{2+}-ATPase in cardiac muscle [14]. However, whereas interaction between PLN and the Ca^{2+}-ATPase is controlled by phosphorylation of PLN, SLN lacks residues that can be phosphorylated; the interaction between SLN and the Ca^{2+}-ATPase is therefore not open to control of the kind observed for PLN. Further, the observation that levels of SLN are very low in some rat fast-twitch muscles [16] shows that the presence of SLN is not essential for Ca^{2+}-ATPase function.

The hydrophobicity of SLN makes its purification from SR very difficult [12]. Studies using SLN extracted from SR have given rather contradictory results. Racker and Eytan [10] reported that co-reconstitution of Ca^{2+}-ATPase and an extract enriched in SLN gave vesicles showing a higher ratio of Ca^{2+} accumulated to ATP hydrolysed than vesicles in the absence of SLN, with a slight decrease in the rate of ATP hydrolysis. Racker and Eytan [10] also reported that addition of SLN to preformed vesicles led to a reduced level of accumulation of Ca^{2+}, with SLN acting as an ionophore. In contrast, MacLennan et al. [28] found that SLN had no effect on Ca^{2+} accumulation when reconstituted with the Ca^{2+}-ATPase.

Here we have studied the properties of synthetic SLN and show that it is readily reconstituted into lipid bilayers with the Ca^{2+}-ATPase. These studies show that the major effect of SLN is to reduce the level of accumulation of Ca^{2+} by the Ca^{2+}-ATPase through effects on leak and slippage pathways.

Incorporation of SLN into lipid bilayers

The fluorescence properties of the single Trp residue at position 23 of SLN can be used to report on the environment of SLN reconstituted into lipid bilayers. The fluorescence emission spectra of SLN reconstituted into di(C14:1)PC or di(C18:1)PC were centred at 326 nm, consistent with a hydrophobic environment for the Trp residue (Figure 1). Quenching of fluorescence by di(Br_2C18:0)PC (Figure 2) was also consistent with a location for the Trp residue in the hydrophobic core of the bilayer. Fluorescence emission spectra for SLN in the longer-chain phospholipids di(C22:1)PC and di(C24:1)PC were also centred at 326 nm, but fluorescence intensities were less than in di(C18:1)PC. A similar observation was made for the peptide $KKGL_7WL_9KKA$ and shown to be due to partial incorporation of the peptide into the bilayer, occurring when the mismatch



Scheme 1 Accumulation of Ca^{2+} by the Ca^{2+}-ATPase

Two conformations of the Ca^{2+}-ATPase are defined as E1 and E2. Binding of Ca^{2+} from the external medium (Ca_{out}) to E1 gives $E1Ca_2$, which can then bind ATP and be phosphorylated to $E2PCa_2$. $E2PCa_2$ can release Ca^{2+} to the inside of the vesicle (Ca_{in}) to give E2P. Hydrolysis then gives the phosphate-bound form $E2P_i$, which releases P_i to regenerate E2. Alternatively, $E2PCa_2$ can release Ca^{2+} to the external medium (Ca_{out}) in the process of slippage to regenerate E2. Passive leak of Ca^{2+} from the inside of the vesicle to the outside is also possible.

Table 3 Effects of SLN on rates of slippage and leak for the reconstituted ATPase

The values for the rate constants for slippage ($E2PCa_2 \rightarrow E2 + 2Ca^{2+}_{out}$) and leak ($Ca^{2+}_{in} \rightarrow Ca^{2+}_{out}$; defined in Scheme 1) were obtained from the simulation shown in Figure 5, and the rate constants for the remaining steps in Scheme 1 were as in Dalton et al. [22]. The molar ratio of di(C18:1)PC/di(C18:1)PA was 9:1.

Lipid	SLN/ATPase molar ratio	Slippage rate constant (s^{-1})	Leak rate constant (s^{-1})
di(C18:1)PC/di(C18:1)PA	0	65	3.0×10^{-6}
	2	130	3.0×10^{-6}
	5	150	1.0×10^{-5}
	10	170	2.5×10^{-5}
	20	300	3.0×10^{-5}
di(C18:1)PC	0	250	1.5×10^{-6}
	5	900	3.0×10^{-6}

between the hydrophobic length of the peptide and the thickness of the bilayer becomes too large [24]. These experiments suggest that the hydrophobic thicknesses of SLN and $KKGL_7WL_9KKA$ are rather similar, consistent with a predicted transmembrane region for SLN of 19 residues. Fluorescence quenching of SLN in mixtures of di(Br_2C18:0)PC and non-brominated phospholipids (Figure 2) is very similar to that for simple peptides of the type $KKGL_nWL_mKKA$, with values for n, the number of sites from which the Trp fluorescence can be quenched, of 2.3 for SLN compared with 2.7 for the simple peptides [24]. Again this is consistent with incorporation of SLN into the lipid bilayers.

Effects of SLN on Ca^{2+}-ATPase activity

Odermatt et al. [14] co-expressed SLN and SERCA1 Ca^{2+}-ATPase in COS cells and studied the Ca^{2+}-dependence of Ca^{2+} accumulation by a microsomal preparation in the presence of oxalate to precipitate Ca^{2+} within the lumen of the microsomes. They reported that the presence of SLN results in a decrease in the pCa value giving a half-maximal rate of accumulation of Ca^{2+} by 0.17 and in a 40% increase in the maximal rate of accumulation of Ca^{2+} [14]. In contrast, when SLN was reconstituted with the Ca^{2+}-ATPase using the procedure used previously for reconstituting the Ca^{2+}-ATPase with PLN [26], SLN was found to have no significant effect on the Ca^{2+} dependence of ATPase activity or on the maximal rate of ATP hydrolysis (Table 1). We conclude that when the rate of ATP hydrolysis is measured directly, SLN, even at the high molar ratio of 5:1 with respect to the Ca^{2+}-ATPase, has no effect on ATPase activity.

Effects of SLN on accumulation of Ca^{2+}

To study the effect of SLN on accumulation of Ca^{2+} driven by the Ca^{2+}-ATPase it was necessary to reconstitute the Ca^{2+}-ATPase into sealed vesicles. The protocol developed by Levy et al. [6] produces vesicles with a very low ionic permeability and high levels of accumulation of Ca^{2+} and, in slightly modified form, was used here. Analysis of samples of ATPase reconstituted with FITC-labelled SLN on sucrose-density gradients showed that Ca^{2+}-ATPase and SLN co-reconstituted (Figure 3). Incorporation of SLN into vesicles of di(C18:1)PC at a 5:1 molar ratio of SLN to ATPase led to a marked decrease in the level of accumulation of Ca^{2+} (Figure 5A). In vesicles of di(C18:1)PC/di(C18:1)PA, where the levels of accumulation of Ca^{2+} are higher [22], the presence of SLN again led to a decrease in the level of accumulation of Ca^{2+}, the effect increasing with increasing molar ratios of SLN/ATPase from 2:1 to 20:1 (Figure 5B).

Accumulation of Ca^{2+} by reconstituted vesicles is a balance between transport of Ca^{2+} into the vesicles by the Ca^{2+}-ATPase, passive leak of Ca^{2+} out of the vesicles down its concentration gradient, and slippage, a process in which the Ca^{2+}-bound, phosphorylated intermediate of the ATPase ($E2PCa_2$) releases Ca^{2+} on the cytoplasmic side of the vesicle rather than on the luminal side, as shown in Scheme 1 [22]. Slippage and leak have distinct effects on the rate of accumulation of Ca^{2+}; in the absence of slippage, the level of Ca^{2+} accumulation increases almost linearly with time until the rate of transport of Ca^{2+} into the vesicles equals the rate of leak outwards, whereas decreased levels of accumulation of Ca^{2+} are seen for short periods of time when slippage is important [22]. As shown in Figure 5, the effects of SLN observed experimentally can be simulated well in terms of the leak/slippage model, using the rate parameters for the ATPase used previously [22] and the rate constants for leak and slippage given in Table 3.

In di(C18:1)PC/di(C18:1)PA, the effect of a 2:1 ratio of SLN/ATPase can be fitted with a 2-fold increase in the rate of slippage with no effect on the rate of leak; higher concentrations of SLN lead to increases in the rates of both slippage and leak. The rate of slippage in di(C18:1)PC is higher than in di(C18:1)PC/di(C18:1)PA, as reported previously [22], accounting for the lower level of accumulation of Ca^{2+}. In di(C18:1)PC, the presence of SLN again increases the rates of both slippage and leak (Table 3). The 3-fold higher rate of leak estimated from the simulations for vesicles containing a 5:1 molar ratio of SLN/ATPase in di(C18:1)PC/di(C18:1)PA compared with those containing no SLN (Table 3) agrees with the $\approx$ 3-fold higher rate of passive leak observed experimentally on removal of ATP from loaded vesicles containing SLN compared with those with no SLN (Figure 9).

Increased rates of slippage and leak in the presence of SLN would be expected to lead to lower concentrations of Ca^{2+} within the lumen of the vesicles. This expectation is consistent with the results of the experiment shown in Figure 6(B). The presence of phosphate in the lumen of the vesicles leads to increased levels of accumulation of Ca^{2+} by precipitating $Ca_3(PO_4)_2$ when the solubility limit of $Ca_3(PO_4)_2$ is exceeded. The presence of luminal phosphate has less effect in the presence of SLN than in its absence (Figure 6B), consistent with a lower level of luminal Ca^{2+} being achieved in the presence of SLN than in its absence.

The effects of SLN do not depend on the method used for reconstitution. We have also reconstituted the ATPase with SLN using the procedure used by Racker and Eytan [10] in which ATPase is mixed with lipid and SLN in cholate, followed by

dialysis to remove the cholate and form vesicles. This procedure gives vesicles that are leaky to Ca^{2+} so that significant accumulation of Ca^{2+} is only observed in the presence of high concentrations of phosphate to precipitate Ca^{2+} in the lumen of the vesicles. As shown in Figure 7(B), the presence of SLN also leads to reduced levels of accumulation of Ca^{2+} for vesicles reconstituted in this way.

A number of experiments were performed to show that the presence of SLN does not lead to breakdown of the lipid bilayer structure or to the formation of non-specific channels in the membrane. The ATPase activities of vesicles containing SLN roughly doubled on addition of the detergent $C_{12}E_8$ to break down the membrane-permeability barrier, showing that reconstitution with SLN resulted in a close to random distribution of ATPase molecules across the two faces of the bilayer, with the bilayer remaining impermeable to ATP (Table 2). The experiments shown in Figure 8 show that the vesicle membrane is impermeable to H^+ in the presence of SLN. This is confirmed by the experiment shown in Figure 6(A); the Ca^{2+}-ATPase acts as a Ca^{2+}/H^+-ATPase [6] and accumulation of Ca^{2+} is increased in the presence of FCCP in either the absence or presence of SLN (Figure 6A), showing that the membrane must have a low permeability to H^+ in both the absence and presence of SLN. These experiments confirm that SLN does not form non-specific channels in the membrane, in agreement with the observations of Shamoo and MacLennan [29] that SLN showed no ion-channel activity in bilayer lipid membranes.

The effects of SLN on accumulation of Ca^{2+} were structurally specific. The simple hydrophobic peptide $KKGL_7WL_9KKA$ had no effect on accumulation of Ca^{2+} (results not shown). Further, PLN had no significant effect on accumulation of Ca^{2+} at a 5:1 molar ratio with Ca^{2+}-ATPase, although at a molar ratio of 50:1 it did decrease the rate of accumulation very significantly (Figure 7A), consistent with its reported inhibition of ATPase activity at high molar ratios of PLN/ATPase [26]. SLN labelled with FITC at a Lys residue introduced at the N-terminus of SLN had the same effect on Ca^{2+} accumulation as SLN (results not shown), suggesting that the N-terminus of SLN is not important for interaction with the ATPase. Since C9A-SLN had the same effect as SLN on the accumulation of Ca^{2+} (Figure 6C), Cys-9 must also be unimportant for function. However, the effects of 3TR-SLN on accumulation of Ca^{2+} were very small (Figure 6C), suggesting that the Thr residues in SLN are important for function. The Thr residues in SLN at positions 5, 13 and 18 are conserved in human, rabbit and mouse SLN. Interestingly, Thr-13 and Thr-18 in SLN are replaced by Cys residues in the corresponding positions of PLN, and mutation of these Cys residues in PLN had no effect on the ability of PLN to inhibit the Ca^{2+}-ATPase, although it did abolish pentamer formation by PLN [26,30].

Effects of SLN on the rate of slippage of the ATPase probably involve direct interactions between SLN and the ATPase, since a change in the rate of slippage implies a change in conformation for the ATPase. The increased rates of passive leak for Ca^{2+} observed at higher concentrations of SLN are also likely to involve interaction with the Ca^{2+}-ATPase, because passive leak of Ca^{2+} from SR vesicles has been suggested to involve the Ca^{2+}-ATPase, since leak is inhibited by thapsigargin, an inhibitor of the Ca^{2+}-ATPase [31]. In the simulations shown in Figure 5, passive leak of Ca^{2+} has been included as a simple, carrier-independent step, $Ca^{2+}_{in} \rightarrow Ca^{2+}_{out}$. The simulations would have exactly the same form if passive leak of Ca^{2+} down its concentration gradient were a carrier-mediated event, involving a complex between the E2 conformation of the ATPase and SLN. However, no detailed simulations have been attempted for this model, since the stoichiometry of the proposed complex between ATPase and SLN is unknown, as is its affinity for Ca^{2+}.

The physiological significance of SLN

The presence in SR of a protein such as SLN that decreases the level of accumulation of Ca^{2+} might seem surprising since SR acts as the main store for Ca^{2+} in muscle cells. However, SLN could have a function in SR related to the role of muscle in non-shivering thermogenesis [32]. In animals lacking brown adipose tissue, the principle source of heat during non-shivering thermogenesis is the hydrolysis of ATP by the Ca^{2+}-ATPase of skeletal-muscle SR [32,33]. Part of the energy released when ATP is hydrolysed is dissipated as heat and part is used to drive Ca^{2+} across the membrane, against its concentration gradient [33,34]. Accumulation of Ca^{2+} is not 100% efficient, and the stoichiometry of Ca^{2+} ions accumulated to ATP molecules hydrolysed is less than 2:1, due to the processes of slippage and leak shown in Scheme 1. These processes of slippage and leak result in the production of heat [31,34]. Since the presence of SLN increases the rates of slippage and leak mediated by the Ca^{2+}-ATPase, it is likely that the presence of SLN will also increase the rate of heat production by SR. A possible role for SLN could therefore be to allow the use of SR in thermogenesis. The SLN content of SR would then be different for different muscle types, depending on the importance of the muscle for thermogenesis. There are, as yet, few estimates of the levels of SLN in different muscle types, but SLN levels are high in skeletal muscle [13] and low in extensor digitorum muscle [16], a peripheral muscle which might be expected to have little role in thermogenesis. It may also be significant that genes for SLN appear to be absent from *Drosophila* and *Caenorhabditis elegans*.

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